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TITLE: Augmentation of a Novel Enzyme/Pro-Drug Therapy "Distant Bystander Effect" to Target Prostate Cancer Metastasis

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Table of Contents

Cover.....	1.....
SF 298.....	2.....
Introduction.....	4.....
Body.....	4.....
Key Research Accomplishments.....	12.....
Reportable Outcomes.....	12.....
Conclusions.....	12.....
References.....	13.....
Appendices.....	14.....

Augmentation of a Novel Enzyme/Pro-Drug Gene Therapy "Distant Bystander Effect" to Target Prostate Cancer Metastasis

Annual Report, DAMD17-01-1-0083, October, 2004

INTRODUCTION:

Prostate cancer is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatments are needed. The subject of this work is a study of gene therapy, used alone and in combination with hormones called cytokines that stimulate the immune system. *The concept is that delivering a cell-killing agent to an accessible site, coupled with help from the immune system can promote reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5FC), is then given, cancer cells that make CDUPRT convert 5FC to a toxin that kills the original cell and others nearby. This system works in slow growing cells like prostate cancer. Killing the cells attracts immune cells. The scope of the work involves identification of the immune cells that infiltrate the prostate when gene therapy is used. We will identify these cells and use cytokines to attract more of them into the prostate. We will then compare the effects of delivering the cytokine gene alone, the suicide gene alone, or a combination of both into mice that carry a murine prostate cancer cell line, RM1 cells, grown in the prostate. We predict that the combination therapy should interfere with the growth of the cancer cells in the prostate and should also cause a reduction in the number and extent of cells that grow in the lung after introduction into the mice via intravenous injection. This work should pave the way for clinical trials of combination therapy involving suicide gene therapy and cytokine gene therapy given together into the prostate of men with prostate cancer.

BODY:

New cell lines were prepared for our studies as described in our report in December, 2003. To test the proof of principle that a combination of gene therapy and immune therapy will target distant as well as local prostate cancer, we decided to use stably transfected cell lines derived from the mouse RM1 line, from T Thompson, Baylor College Texas, rather than using a viral delivery vehicle, thus avoiding any complications resulting from an immune response against the virus. As problems with intellectual property prevented us from continuing our work with the gene, purine nucleoside phosphorylase, for gene-directed enzyme prodrug therapy (GDEPT) directed against prostate cancer (Martiniello *et al.*, 1998; Martiniello-Wilks *et al.*, 2002; Voeks *et al.*, 2002), we have used the fusion gene, cytosine deaminase uracil phosphoribosyl transferase (CDUPRT) as described in our December, 2003 report. CDUPRT has not previously been tested against prostate cancer, making this application novel. The prodrug is 5-fluorocytosine (5FC) which is converted by CD to 5-fluorouridine (5FU). The fusion gene has been shown to be more effective in GDEPT than CD alone, as the UPRT converts 5FU directly to anti-metabolites, 5FdUMP and 5FUTP (Tiraby *et al.*, 1998) and sensitises cancer cells to low doses of 5FU (Kanai *et al.*, 1998). *The drugs generated by CDUPRT can kill both dividing and non-dividing cells.* This is important in prostate cancer, where the percentage of dividing cells is low. Moreover, *metabolites of 5 fluorocytosine can produce a local bystander effect* (Adachi *et al.*, 2000; Pierrefite-Carle *et al.*, 1999) and finally, *CD-GDEPT has been shown to generate a distant bystander effect* against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al.*, 1999).

We also decided that it would be preferable to use cells to carry the cytokine genes of interest into the mice, rather than using lipid-plasmid combinations, which would be less efficient. This would allow us to generate a maximum effect, and so achieve a proof of principle more quickly than by using lipid-based transfection *in vivo*.

Trainee-Fellowship: The work was late in starting because of the intellectual property considerations, and the changes that were necessary to the program. Dr Rosetta Martiniello-Wilks started on the program of work in February, 2003 (instead of September, 2002). However, she left the program in August, to take up a permanent position at another Hospital/ University in Sydney. She was replaced with Dr Bing Zhang, who started in January 2004, but left in June, 2004 because of unforeseeable family considerations. Natalia Liem is now continuing this work. We will be seeking a no-cost extension in order to complete the studies.

Research/Training Please note that Dr Martiniello-Wilks had not previously performed molecular biological work. She received training in the Oncology Research Centre, Prince of Wales Hospital, Department of Clinical Medicine, University of New South Wales; this work is described below.

Reporting: A report of our studies was provided in December, 2003.

Task 1:

Characterization of the extent of the local bystander effect generated by CDUPRT –GDEPT

a, b Development of a stable transfectants of the RM1 cell line

Appropriate plasmids were prepared in pVITRO2-GFP/LacZ and the RM1 cell line was stably transfected under hygromycin (800µg/mL) selection. RM1 cell lines expressing green fluorescence protein (GF) and the genes of interest, i.e., CDUPRT RM1-GFP/CDUPRT (test), (Dec 2003 report, Appendix 1, Fig 1), the murine IL-12 gene (RM1-GFP/IL-12) (Dec 2003 report, Appendix 1, Fig 4), or the murine IL-18 gene (RM1/GFP/IL-18) (Dec 2003 report, Fig 7?) were prepared as described in our previous report. Direct sequencing confirmed the appropriateness of the plasmid inserts (Dec 2003 report, see Appendix II). Control cells were stably transfected to express GFP alone or GFP/LacZ, and were known as RM1-GFP or RM1-GFP/LacZ (controls) respectively. The green fluorescence protein (GFP) was used as a marker to follow the cells *in vivo*, and to sort high expressers as described previously (see Dec 2003 report, Appendix 1, Figures 2, 3, 7). It was also necessary to confirm that RM1 cells did not express receptors for IL-12 or IL-18 (December, 2003 report, Figures 5), so that their growth would not be stimulated by the presence of the cytokines.

Effects of 5FC on RM1 cells.

First it was necessary to determine that the prodrug, 5FC, was not toxic to RM1 cells or to the host mice, and that the RM1 cells were sensitive to treatment with 5FU (the final product).

RM1 cells were grown by subcutaneous injection of C57BL/6 mice with 2.5×10^6 cells. The mice were then treated with daily intraperitoneal injections of 5FC at three different doses for 13 days. After this time, the mice were sacrificed, and various organs taken for histological assessment. In addition, serum was collected and analysed for presence of either the prodrug or its metabolite 5FU. The serum was analysed using the HPLC (see below for details) and there were no detectable levels of either 5FC or 5FU in the serum from the treated mice at day 14 of the treatment.

The results in Table 1 show that the 5FC treatment had no adverse effects on liver or kidney biochemistry, and photomicrographs of H&E stained sections of heart, kidney, liver or spleen, (DOD report, October 2004, Figure 1) indicate that there were no toxic effects seen in the treated mice.

In contrast to the results obtained with 5FC, RM1 cells were killed by exposure to 5FU for one week *in vitro* (DOD report, October 2004, Figure 2).

Table 1: Effects of treatment of RM1- bearing C57BL/6 mice with 5FC

Treatment	Urea (mmol/L)	Creatinine (umol/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
Reference	1.4-5.5	18-80	35-96	17-77	54-298
Control	6.7 ± 0.3	29.3 ± 3.3	54 ± 1.7	32.5 ± 5.7	155 ± 25
150 mg/kg/d	6.8 ± 0.4	20 ± 1.5	67 ± 2.5	27 ± 3.1	92.8 ± 16.5
300 mg/kg/d	6.7 ± 0.3	26.5 ± 0.9	62.3 ± 1.7	18.3 ± 1.8	68.5 ± 13.9
500 mg/kg/d	6.8 ± 0.7	25.3 ± 1.8	55.7 ± 4.9	19 ± 1.5	57.3 ± 11.9

Footnote:

AST (Aspartate Aminotransferase, Also known as Serum Glutamic Oxaloacetic Transaminase): This enzyme is found primarily in the liver, heart, kidney, pancreas, and muscles. It is generally elevated when there is tissue damage, especially to the heart and liver.

Alkaline phosphatase (ALP): Produced in the cells of the bone and liver with some activity in the kidney, intestine, and placenta. Used extensively as a marker, it is also present in bone injury, pregnancy, or skeletal growth (elevated values). Low levels are sometimes found in hypoadrenia, protein deficiency, malnutrition, and a number of vitamin deficiencies.

Urea (Blood Urea Nitrogen; BUN): The nitrogen component of urea, BUN is the end product of protein metabolism and its concentration is influenced by the rate of excretion. Increases can be caused by excessive protein intake, kidney damage, certain drugs, low fluid intake, intestinal bleeding, exercise, or heart failure. Decreased levels may be due to a poor diet, malabsorption, liver damage, or low nitrogen intake.

Creatinine: Creatinine is the waste product of muscle metabolism. Its level reflects the body's muscle mass. Low levels may be seen in kidney damage, protein starvation, liver disease, or pregnancy. Elevated levels are sometimes seen in kidney disease, muscle degeneration, and some drugs that impair kidney function.

Alanine transaminase (ALT): Also known as serum glutamate pyruvate transaminase (SGPT) This test is used to determine damage to the liver. ALT is involved in the alanine metabolism, is found in a number of tissues but is in highest concentration in the liver. Injury to the liver results in release of the enzyme into the blood

CDUPRT expression by transfected cells:

In vitro: The stably transfected RM1-GFP/CDUPRT cells were examined to show that the genes of interest were functional. An HPLC based assay tested the capability of the cell/ lysates to catabolise the prodrug 5FC to 5FU. Specifically, 10⁷ RM1-GFP/CDUPRT /RM1-GFP/LacZ cells were resuspended in 110 µL of PBS and lysed by repeat freeze thawing of the cells by alternating between incubation in liquid Nitrogen and a 37°C waterbath. The cell debris was removed by centrifugation of the lysates at 12,000g for 10 minutes. The protein content of the supernatants was determined using the BCA protein estimation assay kit (Pierce, Rockford, IL, USA). 100µL of the supernatant was then incubated with 900µL of 0.5 mM 5-FC at 37°C (waterbath) for different time periods (1, 6.5 and 24 h). At each time point 100µL of the samples were harvested and incubated at 85°C for 10 minutes, then stored at -20°C until analysis. The samples were analysed using reverse phase chromatography. Sample (10µL) was injected into a C18 column under isocratic conditions (0.05% Trifluoroacetic acid in water) at a flow rate of 0.7 mLs/min. Absorbance was measured at 275 nm. The enzyme activity for each sample was determined by ratios of the peak areas for 5FC and 5FU. High enzymic activity was measured over a 24 h period (October 2004 report, Appendix

1, **Figure 3A**). These data confirmed our previous findings (December 2003 report, Appendix 1, Figure 8), which showed that RM1-GFP/CDUPRT cells treated with 5FC above 3 µg/mL showed a marked reduction in proliferation compared with control cells.

In vivo: RM1-GFP/CDUPRT (2.5×10^6) cells were injected subcutaneously in C57BL/6 immuno-competent male mice and the tumors were harvested when they reached the size 10x10mm. Control mice received RM1-GFP/LacZ cells. The tumors were homogenized in PBS using liquid nitrogen followed by three repeat cycles of freeze (liquid nitrogen)-thawing (37°C) to complete the cell lysis. The cell debris was removed by centrifugation @ 15,000g for 10 minutes and the homogenates examined for CDUPRT enzymic activity by the HPLC assay as described above (October 2004 report, Appendix 1, **Figure 3B**). Effective production of 5FU was demonstrated.

The sensitivity of the HPLC-based assay was also evaluated and the minimum protein concentration at which the 5FC-5FU conversion was detectable by HPLC was 0.3 mg (data not shown). This is equivalent to $\sim 2 \times 10^6$ cells in *in vitro* tissue culture. Furthermore, comparison of tumor samples with the cultured cells showed that at 24 h, 6 fold more protein was required from the tumor samples to achieve the same sensitivity. This could be accounted for by the presence of non-tumor tissue in the tumor sample such as stroma and vasculature (Data not shown).

GDEPT effected by RM1-GFP/CDUPRT cells plus 5FC

Based on the above, the maximum non-toxic dose of prodrug that we could solubilise and use daily was 500mg/kg/day. We first established that RM1-GFP/CDUPRT cells could effect GDEPT *in vivo*. C57BL/6 mice were implanted in the prostate with 5×10^3 of RM1-GFP/CDUPRT (test) or RM1-GFP/LacZ (control) cells, and 4 days later the prodrug 5FC (or saline, as a control) was administered intraperitoneally at 500mg/kg/day for 14 days. On day 18, mice were sacrificed and their prostate volumes determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles. GDEPT from the use of RM1-GFP/CDUPRT cells plus 5FC was extremely effective, with almost complete absence of growth in the prostate compared with control cells, or with RM1-GFP/CDUPRT treated with saline (October 2004 report, Appendix 1, **Figure 4**).

Tumors were taken from mice treated with RM1-GFP/CDUPRT with saline (6 mice) or with 5FC (10 mice) and examined histologically after H & E staining. RM1-GFP/CDUPRT tumors from mice given saline all showed >80% viability, with <10% necrosis or haemorrhagic necrosis, whereas those from mice given daily 5FC had no tumor (in 6 cases), or <10% viable tumor (4 cases). In the latter cases, there was >30% necrosis, and >60% haemorrhagic necrosis (Table 2). A comparison of these tumors is shown in the October 2004 report, Appendix 1, **Figure 5**. Other tissues were taken for histological examination including kidney, lung, spleen, liver, but no abnormal findings were seen in any of the mice whether treated with saline or 5FC. This indicated that the effects were specific to the tumor, and no other toxicity was observed. Very little infiltrate was seen in any case.

Table 2: Effects of CDUPRT-GDEPT on prostate histology.

Treatment 4.5 x RM1-GFP/CDUPRT cells injected into the prostate	viability	necrosis	Haemorrhagic necrosis
+ saline ip 15 days (n=6)	>80%	<10%	<10%
+ 5FC ip daily (n=4)	<10%	>30%	>60%
+ 5FC ip daily (n=6)	No	No growth	No growth

Local bystander effect on RM1 growth.

Any local bystander effects from CDUPRT-GDEPT were assessed *in vitro* and *in vivo*. To overcome the problem that different cell lines grew at different rates, we tested conditioned media from RM1-GFP/CDUPRT cells (grown with or without 5-FC (at 1mM) or RM1-GFP/LacZ cells (with 5-FC) on the growth of RM1 parental cells. The media were harvested 48 h after the addition of 5-FC. RM1 parental cells were plated at 5×10^3 cells/well in a 96 well plate, and media conditioned by the different cells were mixed with an equal proportion of fresh medium. Results in October 2004 report, Appendix 1, **Figure 6A** show that medium collected from RM1-GFP/CDUPRT with 5FC but not RM1-GFP/LacZ cells (with 5-FC) prevented the growth of RM1 cells.

To assess any local bystander effect of GDEPT treatment on RM1 growth, RM1-GFP/CDUPRT cells and RM1-GFP/LacZ cells were mixed in different proportions for inoculation of mice, that were then treated with 5FC for 14 days at 500mg/kg/day. The minimal proportion of RM1-GFP/CDUPRT cells required to produce a therapeutic effect were thus established and found to be 20% (October 2004 report, Appendix 1, **Figure 6B**).

Distant bystander effects of CDUPRT-GDEPT

To determine whether killing of cells in the prostate by CDUPRT-GDEPT would have any effect on the growth of pseudometastases of RM1 cells in the lung, the following experiment was performed. C57BL/6 mice were injected intraprostatically with 5×10^3 RM1-GFP/CDUPRT or RM1-GFP cells. Four days later, mice received 2.5×10^5 RM1 cells intravenously, and were treated with 500 mg/kg/day 5FC daily ip for 15 days. Mice were sacrificed on day 19 and their lungs removed, fixed in Bouin's fixative, and the colonies caused by RM1 growth were counted. The results in **Figure 7** (October 2004 report, Appendix 1, **Figure 7**) show that the number of lung colonies in mice given RM1-GFP/CDUPRT in their prostate was much lower than that in mice given RM1-GFP cells. Thus 50% of mice given RM1-GFP/CDUPRT and treated with 5FC had no lung colonies, whereas those treated with RM1-GFP/CDUPRT and saline, and control mice (that received RM1-GFP cells + 5FC) had no mice in this category. In contrast, >75% mice treated with RM1-GFP/CDUPRT + saline and ~60% of mice that received RM1-GFP cells + 5FC had >100 colonies/lung. This suggests that an immune response, generated by the release of tumor antigens when RM1 cells were killed by GDEPT may have prevented the growth of lung colonies of RM1 cells injected intravenously. It is also possible that 5FU released by conversion of 5FC by CDUPRT in the prostate may have had a direct effect on lung colonisation of RM1 cells, and we will perform further experiments to test this possibility.

Task 2: Characterisation of immune cells responsible for the GDEPT distant bystander effect

We have characterised the growth of the RM1-FP/mIL12 and RM1-GFP/mIL18 cells in C57BL/6 mice as described below.

Gene expression by RM1-GFP/mIL12 and RM1-GFP/mIL18 cells:

1. Protein detection assay: mIL-12 and mIL-18 expression by RM1-GFP/mIL12 or RM1-GFP/mIL18 cells was examined by ELISA (BD Pharmingen) but the assay was not successful for cell supernatants. As an alternative, a western blot based assay is currently being optimised. The preliminary data obtained thus far indicate the presence of secreted cytokines in both cell lines (**data not shown**).

2. Functional assay: This was developed to assess the functionality of the cytokines produced by these cells lines.

To minimise the use of animals as spleen cell donors for the traditional functional assays for IL12 and IL18, an assay based on the use of murine CTLL2 cells was developed. The rationale for the assay was based on the following two facts:

1. At least 60% of CTLL2 cell population has mIL12 receptors as shown by the data obtained from FACs-based analysis of immunostaining of CTLL2 cells with mIL12 receptor antibody. (see December 03 report, Appendix I).
2. IL12 and IL18 synergise with each other by upregulating each other's receptors. IL-12 can upregulate the production of the IL-18 receptor α (IL18R α) chain on Th1 cells (Ahn *et al.*, 1997) whereas IL-18 has been shown to upregulate the IL12R β 2 chain on Th1 cells (Chang *et al.*, 2000). This shared upregulation of receptors provides a positive feedback mechanism allowing these cytokines to act synergistically.

Method: CTLL2 cells (obtained from Dr Peter Williamson's laboratory from S Schibeci at Millennium Institute at Westmead Hospital, Sydney) are derived from C57BL/6 mice and grow indefinitely in culture in the presence of 20u/mL of mIL2 (Roche). In general, 5×10^3 cells were seeded in a 96-well plate and at the relevant times post treatment, the proliferation was measured by 4 h incubation of the cells in WST1 (10 μ l/100 μ l, Scientifix). As the assays were being developed to examine the proliferative effects of mIL12 and mIL18 on CTLL2 cells, the normal doses of mIL2 (20u/ml, Roche, USA) would have masked the effects of other cytokines. The aim of the first set of experiments was to determine a suboptimal concentration of mIL2 that would allow the cells to survive but not to proliferate. Cells were incubated with different doses of mIL2 (50-0 units/ml; October 2004 report, Appendix I, **Figure 8**) and analysed for proliferation at 24, 48 and 72 h. *These data suggested that at 2u/L these cells were able to survive until 72h without proliferating.* From this point on all assays were done using assay media containing mIL2 at 2u/mL. Furthermore, the integrity of the cells was determined by the proliferative response of CTLL2 cells to mIL2 in all experiments.

Experiment 1: *CTLL2 cells respond to the presence of recombinant mIL12 and mIL18 in the growth medium.*

Cells were incubated with recombinant mIL12 (R&D systems, USA) or mIL18 (Medical and Biological Laboratories Co., LTD, Japan) (ranging from 0-100 ng/ml) for 72 hrs. The cells showed a clear proliferative response to both cytokines. The response was enhanced by 3.2 fold when treated with mIL12 (100ng/ml, October 2004 report, Appendix I, **Figure 9A**) and by 3.7 fold when treated with mIL18 (0.1 ng/ml; **Figure 9B**).

Experiment 2: *CTLL2 cells respond to the supernatants from RM1-GFPmIL12 and RM1-GFPmIL18.* Cells were incubated with supernatants at concentrations ranging from 100% to 0% from RM1-GFPmIL12 and RM1-GFPmIL18 cells, respectively. Since at the moment we have no conclusive estimate of the quantities of the cytokines produced by these cell lines, a % of the supernatants was applied. As a control, supernatant from RM1-GFP cells was used in both cases. Measurement of the absorbance (at 450nm) at 72 h post treatment clearly showed that proliferation was enhanced 4.7-6.9 fold enhancement by RM1-GFPIL12 supernatant compared to the RM1-GFP controls (October 2004 report, Appendix I, **Figure 10A**). However, even though the proliferative response of CTLL2 cells to supernatants from RM1GFPmIL18 was obvious (~2.5 fold at 12.5 and 6.25%; October 2004 report, Appendix I, **Figure 10B**), it was not as significant as that observed with mIL12 containing supernatants.

Experiment 3: *CTLL2 proliferation is further enhanced when treated with both mIL12 and mIL18 in tandem.* As the proliferative response of the CTLL2 cells to mIL18 was not as strong as that observed with mIL12, this experiment was designed to enhance the proliferative effects of the two cytokines by using them in combination. *The hypothesis was that initial incubation with mIL12 would upregulate the mIL18 receptors in CTLL2 cells and this would lead to an enhanced response of the CTLL2 cells to mIL18 as a follow up treatment (refer above for the relevant references).* Cells were treated with a range of concentrations of mIL12 (0-100ng/ml) and after 24 h incubation

mIL18 was added to the cultures at varying concentrations (0-100ng/ml). Cells treated with both cytokines together showed an enhanced proliferation compared with either alone. Specifically, this enhancement was significant at higher concentrations of mIL12 (10 and 100 ng/ml) (October 2004 report, Appendix I, **Figure 11**). Maximal effects were observed at the combination of mIL12 at 10ng/ml with mIL18 at (1,10 or 100ng/ml). Significantly, the enhancement was ~10 fold at mIL12 at 10ng/ml and mIL18 at 100ng/ml.

The experiments described above have shown that:

1. CTLL2 cells respond to both mIL12 and mIL18.
2. RM1-GFPmIL12 and RM1-GFPmIL18 secrete functional cytokines.
3. The proliferative response of the CTLL2 cells is further enhanced when the two cytokines are used in combination.

To further support these observations we plan to show conclusively that these effects are mediated by the cytokines by using the relevant neutralising antibodies to the two cytokines.

Note: This functional assay has not only facilitated the assay of the two cytokines without the use of animals in this laboratory, but also the data generated are sufficient to submit as a technological note to Journal of Immunological Techniques. This paper is in preparation.

In vivo studies using RM1-GFP/mIL-12 cells: In order to investigate the effects that IL-12 expression alone has on growth and to determine a suitable cell number for future RM1-GFP/IL12 implantations, C57BL/6 mice were initially injected sub-cutaneously (sc) with either RM1-GFP/IL12 cells or RM1-GFP/LacZ control cells at 1.5×10^4 , 1.5×10^5 or 1.5×10^6 cells/mouse. Tumor growth was measured on days 4, 7 and 14. Tumor samples were harvested on day 14 and stored embedded in OCT media at -80°C or in paraffin at room temperature.

Table 3: Take rate of RM1-GFP/mIL12 and RM1-GFP/LacZ cells in C57BL/6 mice.

Cells Implanted	Day 7 Take Rate	Day 14 Take Rate
1.5×10^4 RM1-GFP/LacZ	0%	33%
1.5×10^5 RM1-GFP/LacZ	100%	100%
1.5×10^6 RM1-GFP/LacZ	100%	100%
1.5×10^4 RM1-GFP/IL12	0%	40%
1.5×10^5 RM1-GFP/IL12	66%	66%
1.5×10^6 RM1-GFP/IL12	0%	60%

At cell numbers of $\geq 1.5 \times 10^5$ cells, RM1-GFP/IL12 cells had both a lower take rate (60-66% compared with 100%) (Table 2) and slower growth than equivalent numbers of RM1-GFP/LacZ cells treated similarly (October 2004 report, Appendix I, **Figure 12A**). Moreover increasing the number of cells injected caused slower growth (lower average tumor volume, October 2004 report, Appendix I, **Figure 12A**) and a lower take rate (1.5×10^6 RM1-GFP/IL12 cells 60% take rate vs. 1.5×10^5 RM1-GFP/IL12 cells, 66% take rate, Table 2). Thus mice injected with 1.5×10^6 cells showed no detectable tumors by Day 7, in contrast to those receiving 1.5×10^5 RM1-GFP/mIL12 cells, or mice injected with 1.5×10^5 cells and 1.5×10^6 cells RM1-GFP/LacZ cells. These data suggest that the mIL12 was biologically active and secreted, inhibiting tumor take and growth. A second experiment confirmed that the growth of RM1-GFP/IL12 cells was slow and achieved a smaller volume (note different scales on the X axis, **Figure 12B**) than parental RM1 or control RM1-GFP cells. Sections of the tumors taken at sacrifice were stained with H&E to assess immune cell infiltration. When examined histologically sections from sc RM1-GFP/IL12 tumors showed more extensive necrosis and immune cell infiltration than those from RM1-GFP/LacZ s (data not shown).

Similarly, when RM1-GFP/IL18 cells (3×10^5) were inoculated sc into C57BL/6 mice, the growth of the cells was extremely slow and the tumor size achieved was much smaller compared with that of parental RM1 or control RM1-GFP cells (October report, Appendix I, **Figure 12C**; note differences in X axes). In rare cases, rapid growth of a given tumor suddenly occurred suggesting outgrowth of a modified clone of cells.

When RM1-GFP/IL12 or RM1-GFP/IL18 cells were injected into the prostate of C57BL/6 mice, the volume of tumor growth appeared to be related to cell number (DOD report, October 2004, Appendix 1, **Figure 13**), but histological examination indicated that tumors arising after injection of larger cell numbers showed decreased viability, with increased haemorrhagic necrosis than those from injection of lower cell numbers (Table 4). The following parameters were assessed: extent of vascularisation, % Viable tumor; % Tumor necrosis (assessed as pyknotic necrosis (with dense chromatin) at the margin of viable tumors or coagulative necrosis (ghost-like cells) further away from the blood vessels, and % haemorrhagic necrosis.

Table 4: Histological changes in tumors arising in the prostate after injection of RM1-GFP/IL12 or RM1-GFP/IL18 cells.

<u>Treat- ment group no</u>	<u>Mouse no</u>	Tumor cells injected	Prostate tumor size (mm)	Histology			
				Vascu- larity	% Haemorr- -hagic necrosis	% Tumour Necrosis	% Viable Tumour
RM1- IL12 #1	168.1	4.5×10^3	10x7	High	>2	>5	<80
	168.2	4.5×10^3	10x10	High	>2	>10	<80
	191.2		NA				
RM1- IL12 #2	189.2	4.5×10^4	6x4	High	>?	>5	<80
	190.1	4.5×10^4	12x12	High	>10	>35	<50
	190.3	4.5×10^4	8x7		>20	>35	<40
RM1- IL12 #3	187.1	4.5×10^5	13x10		>20	>30	<40
	187.3	4.5×10^5	6x4		>40	>40	<10
	188.2	4.5×10^5	15x15		>40	>40	<20
RM1- IL18	182.1	?	10x6	Mod- erate	>20	>30	<35

In addition, frozen sections of the orthotopic tumors arising from injection of RM1-GFP/IL12 cells were examined for infiltrating mouse immune cells, by staining with Rabbit-anti Asialo GM1 (*Wako Pure Chemical Industries* C/N 986-10001, diluted 1/00), that detect NK cells, CD4, CD8 (BD Pharmingen, C/N 550281, diluted 1/200) and CD90 (CD90.2 (Thy 1.2) BD Pharmingen C/N 550543, diluted 1:400) that detect T cells, and F4/80 (BD Pharmingen, C/N 552958, diluted 1/800) that detects macrophages. Anti-rat and anti-rabbit antibodies were used as a secondary-linked antibodies at the optimal concentrations as shown: Biotinylated rabbit anti-rat: 1:200 (for CD8a, CD90 and F4/80), Vector Lab C/N I0813 and Biotinylated goat anti-rabbit: 1:1000 (for Asialo GM1), Vector Lab, C/N K0323.

Intraprostate tumors arising from injection of control cells, RM1 or RM1-GFP contained no CD8 cells, or F4/80 positive cells, but there were few asiolo-GM1 positive cells, and some CD90 positive cells around blood vessels. In contrast s from RM1-GFP/IL12 cells showed an increase in infiltrating immune cells, in particular NK (Asialo-GM1 positive cells, seen in clusters), both CD8 positive (few) and CD90 positive (especially around blood vessels and in haemorrhagic necrotic areas) and some F4/80 positive cells (see 2004 October report, Appendix I, **Figure 14**).

In our next experiments, we will complete Task 2, by investigating whether anti-prostate cancer activity can be identified in infiltrating lymphocytes within the prostate after CDUPRT-GDEPT, or after injection of RM1-GFP/IL12 or IL18 cells, and examine the effects of immune cell depletion on these activities. We will then be in a position to begin Task 3.

KEY RESEARCH ACCOMPLISHMENTS:

- Established stably transfected murine prostate cancer lines from RM1 that express the transgenes and the reporter gene, green fluorescence protein: RM1-GFP/CDUPRT cell line; RM1-GFP/mIL-12 and RM1-GFP/mIL18 cell lines.
- Established and tested assay systems to measure expression of the transgene, CDUPRT *in vitro* and *in vivo*.
- Established local and distant bystander effects of CDUPRT-GDEPT system in RM1 tumors *in vivo*.
- Measured functionality of expression of the IL-12 and IL-18 transgenes against CTLL2 cells
- Performed preliminary work to determine the growth rate of RM1-GFP/IL12 and RM1-GFP/IL18 lines grown subcutaneously or in the prostate of C57BL/6 mice *in vivo*.

REPORTABLE OUTCOMES:

- Establishment of new cell lines derived from RM1: RM1-GFP/CDUPRT; RM1-GFP/mIL-12; RM1-GFP/mIL-18.
- Dr Rosetta Martiniello-Wilks has been appointed as a Senior Hospital Scientist at Royal Prince Alfred Hospital to set up a GLP facility for Gene Therapy trials. She was the successful candidate for this position because she was a DOD Trainee-fellow.
- We are in the position to write a paper on the bystander effects seen when CDUPRT cells are injected into the prostate of C57BL/6 mice. This manuscript is in preparation.
- We will also write a technical paper about the use of CTLL2 cells for examining the effects of mIL12 and mIL18 and the synergy between these two cytokines.

CONCLUSIONS

We have shown that gene directed enzyme prodrug therapy directed by CDUPRT plus 5FC can eliminate the growth of RM1 prostate cancer cells in the prostate of C57BL/6 mice, and is associated with an excellent local bystander effect, both *in vitro* and *in vivo*, as well as a distant bystander effect. Thus local therapy given into the prostate inhibits the growth of pseudometastases induced by the intravenous administration of RM1 cells.

We have also shown that mIL12 and mIL18 given alone can inhibit the growth of RM1 cells implanted subcutaneously or in the prostate of C57BL/6 mice.

We have established a new model for examining the effects of mIL12 and mIL18 against CTLL2 cells grown *in vitro*, obviating the need to assess their functionality by *in vivo* experiments. We have demonstrated synergy between these two cytokines in their anti-proliferative effects against CTLL2 cells *in vitro*.

REFERENCES:

- Adachi Y, Tamiya T, Ichikawa T, Terada K'Y, Ono Y, Matsumoto K, Furata T, Hamada H, Ohmoto T. Experimental gene therapy for brain s using adenovirus-mediated transfer of cytosine deaminase gene and uracil phosphoribosyltransferase gene with 5-fluorocytosine. *Human Gene Ther* 2000;11:77-89.
- Ahn JH, Maruo S, Tomura M, Mu J, Hamaoka T, Nakanishi K, Clark S, Kurimoto M, Okamura H, Fujiwara H. A mechanism underlying synergy between IL-12 and IFN- γ -inducing factor in enhanced production of IFN- γ . *J Immunol* 1997;159:2125-2131.
- Chang JT, Segal BM, Nakanishi K, Okamura H, Shevach EM. The costimulatory effect of IL-18 on the induction of antigen-specific IFN- γ production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor β 2 subunit. *Eur J Immunol* 2000;30:1113.
- Chung-Faye GA, Chen JM, Green NK, Burton A, Anderson D, Mautner V, Searle PF, Kerr DJ. In vivo gene therapy for colon cancer using adenovirus-mediated transfer of the fusion gene, cytosine deaminase and uracil phosphoribosyltransferase. *Gene Therapy* 2001;8:1547-54.
- Kanai F, Kawakami T, Hamada H, Sadata A, Yoshida Y, Tanaka T, Ohashi M, Tateishi K, Shiratori Y, Omata M. Adenovirus-mediated transduction of Escherichia coli uracil phosphoribosyltransferase gene sensitises cancer cells to low concentrations of 5-fluorouracil. *Cancer Res* 1998;58:1946-1951.
- Koyama F, Sawada H, Hirao T, Fujii H, Hamada H, Nakano H. Adenoviral-mediated transfer of Escherichia coli uracil phosphoribosyltransferase (UPRT) gene to modulate the sensitivity of the human colon cancer cells to 5-fluorouracil. *Eur J Cancer* 2000;36:2403-2410.
- Martiniello-Wilks R**, Garcia-Aragon J, Daja M, Russell P, Both GW, Molloy PL, Lockett LJ, **Russell PJ**. In vivo gene therapy for prostate cancer: preclinical evaluation of two different enzyme-directed prodrug therapy systems delivered by identical adenovirus vectors *Human Gene Therapy*, 1998; 9:1617-1626.
- Martiniello-Wilks R**, Tsatralis T, Russell P, Brookes DE, Zandvliet D, Lockett LJ, Both GW, Molloy PL and **Russell PJ**. Transcription-targeted gene therapy for androgen-independent prostate cancer. *Cancer Gene Therapy*, 2002;9:443-52.
- Nishiyama T, Kawamura Y, Kawamoto K, Matsumura H, Yamamoto N, Ito T, Ohyama A, Katsuragi T, Sakai T. Antineoplastic effect in rats of 5-fluorocytosine in combination with cytosine deaminase capsules. *Cancer Res* 1985;45:1753-1761.
- Perrefite-Carle V, Baque P, Gavelli A, Mala M, Chazal M, Gugenheim J, Bourgeon A, Milano G, Staccini P, Rossi B. Cytosine deaminase/5-fluorocytosine-based vaccination against liver s: Evidence of distant bystander effect. *J Natl Cancer Inst* 1999;91:2014-19.
- Tiraby M, Cazaux C, Baron M, Drocourt D, Reynes J-P, Tiraby G. Concomitant expression of *E. coli* cytosine deaminase and uracil phosphoribosyltransferase improves the cytotoxicity of 5-fluorocytosine. *FEMS Microbiol Letters* 1998;167:41-9.
- Voeks D, **Martiniello-Wilks R**, Madden V, Smith K, Bennetts E, Both GW, and **Russell PJ**. Gene therapy for prostate cancer delivered by ovine adenovirus and mediated by purine nucleoside phosphorylase and fludarabine in mouse models. *Gene Ther*, 2002; 9(12): 759-768.
- Yoshimura I, Ikegami S, Suzuki S, Tadakuma T, Haykawa M. Adenovirus mediated prostate specific antigen promoter enhanced by the cre-loxp system. *J Urol* 2002;168:2659-2664.

Appendix I

Figure 1: Photomicrographs of tissues indicating lack of toxicity of 5-fluorocytosine in C57BL/6 mice

Figure 2: Kill curve generated by treatment of parental RM1 cells with different doses of 5-fluorouracil (5FU)

Figure 3: Characterisation of RM1-GFP/CDUPRT cells. A. *in vitro*; B *in vivo*

Figure 4: GDEPT effected by RM1CDUPRT cells *in vivo*.

Figure 5: Effects of CDUPRT-GDEPT on histology of RM1 tumors

Figure 6A: *In vitro* evaluation of the local bystander effect of CDUPRT-GDEPT.

Figure 6B: Local bystander effect of CDUPRT-GDEPT *in vivo*.

Figure 7: Distant bystander effect of CDUPRT-GDEPT *in vivo*.

Figure 8: Proliferation of CTLL2 cells in response to mIL2 at 72 hr post-treatment.

Figure 9: CTLL2 proliferation in response to recombinant mIL12 (A) and mIL18 (B).

Figure 10: CTLL2 proliferation in response to stimulation by supernatants from RM1-GFPmIL12 (A) and RM1-GFPmIL18 cells (B). Supernatants from RM1-GFP cells were used as the controls.

Figure 11: CTLL2 proliferation in response to combination of recombinant mIL12 and mIL18. Cells were incubated with mIL12 for 24 hrs followed by addition of mIL18, and then analysed for proliferation 48 hrs later. The data are normalised for no addition of mIL12 or mIL18

Figure 12: Growth of RM1-GFP/IL12 or RM1-GFP/IL18 cells sc in C57BL/6 mice

Figure 13: Relative growth of RM1-GFP/IL12 and RM1-GFP/IL18 cells in the prostate of C57BL/6 mice after orthotopic implantation. RM1-GFPmIL12 (A) and RM1-GFPmIL18 cells (B). Supernatants from RM1-GFP cells were used as the controls.

Figure 14: Photomicrographs of immune reactive cell infiltration (x10) in RM1 tumors expressing IL12.

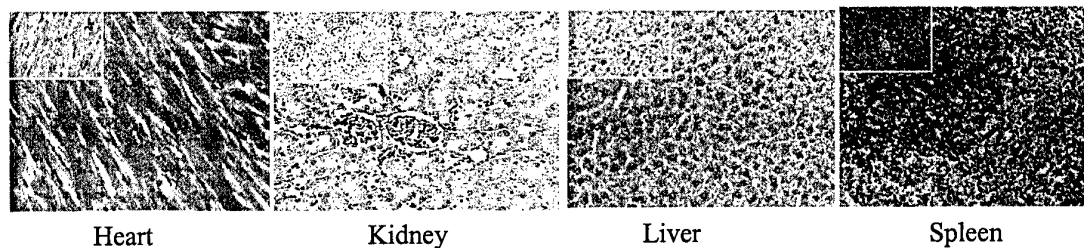


Figure 1: Photomicrographs of tissues indicating lack of toxicity of 5fluorocytosine (5FC) in C57BL/6 mice

After treatment for 15 days with 5FC at 500mg/kg/day, given intraperitoneally, mice were sacrificed and their organs, heart, kidney, liver and spleen were formalin-fixed and paraffin-embedded. Sections stained with H&E were compared with those from control untreated mice (shown as inserts). No toxic effects were seen.

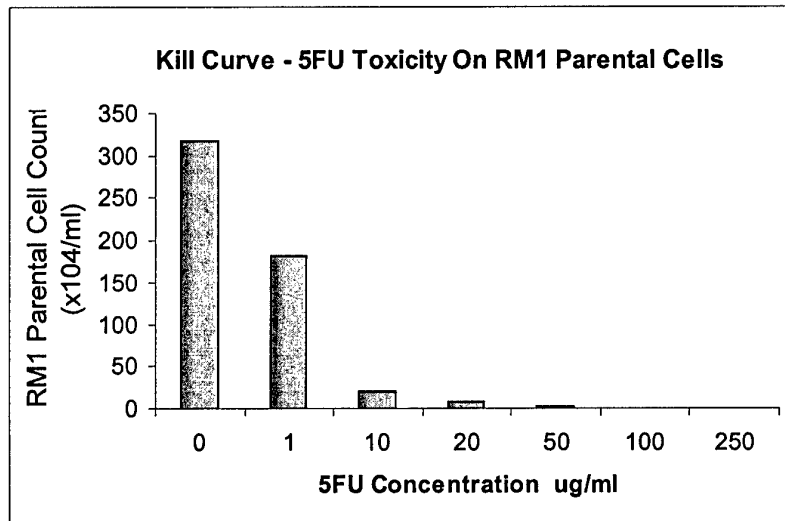


Figure 2: Kill curve generated by treatment of parental RM1 cells with different doses of 5FU.
Cells were under treatment for one week.

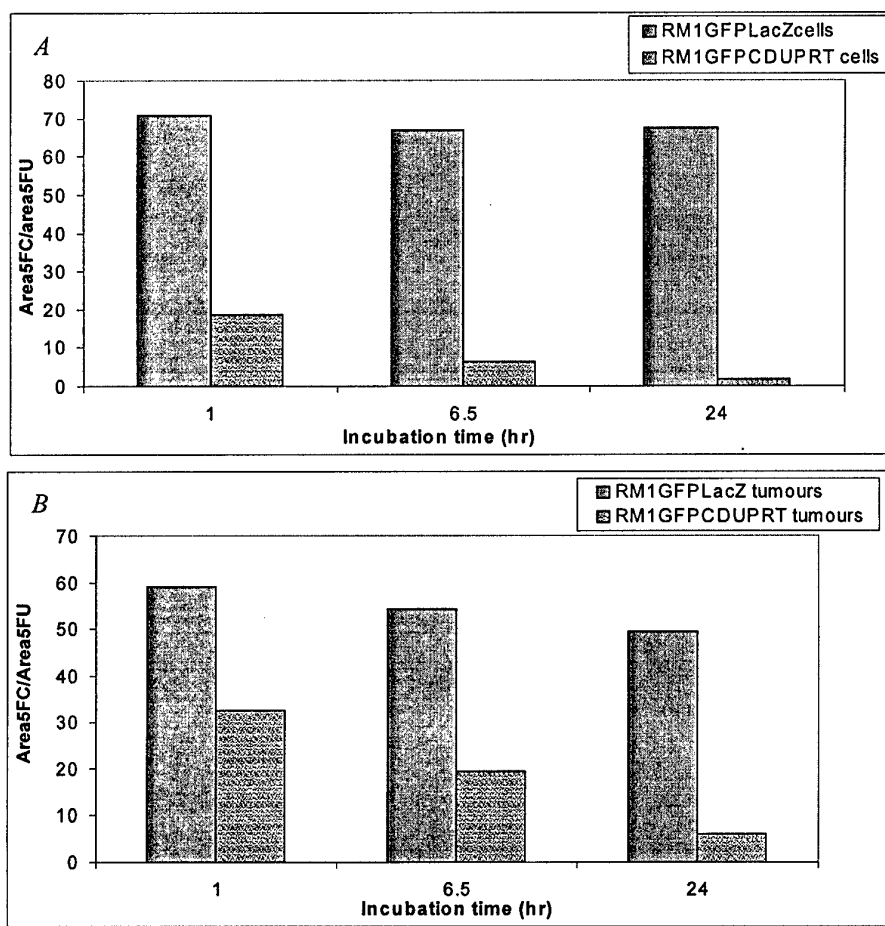


Figure 3: Characterisation of RM1-GFP/CDUPRT cells.

RM1-GFP/CDUPRT cells were tested for CDUPRT expression. The assay involved HPLC based evaluation of the capability of the cell/tumour lysates to catabolise the prodrug 5FC to 5FU. The enzyme activity for each sample was determined by ratios of the peak areas for 5FC and 5FU. Panel A shows the activity of the enzymes in lysates derived from RM1-GFP/CDUPRT cells *in vitro* and panel B shows the activity of the enzyme in lysates generated from the RM1-GFP/CDUPRT tumours grown subcutaneously in C57Bl/6mice. RM1-GFP/LacZ cells or tumours served as controls.

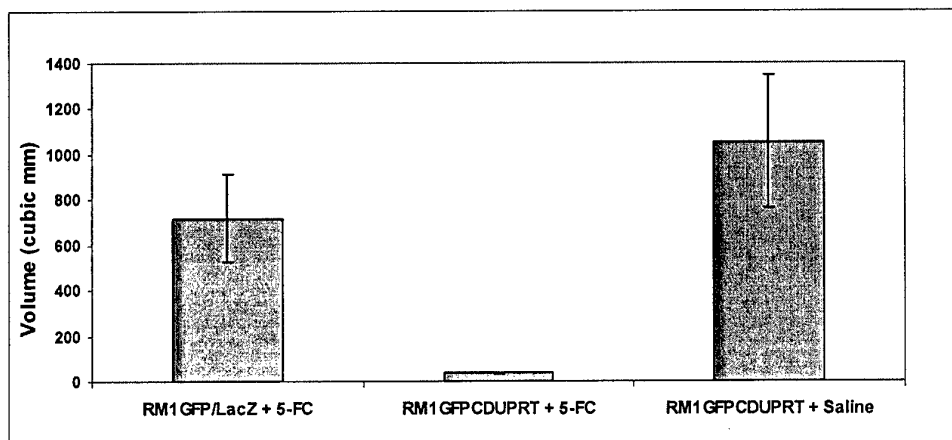
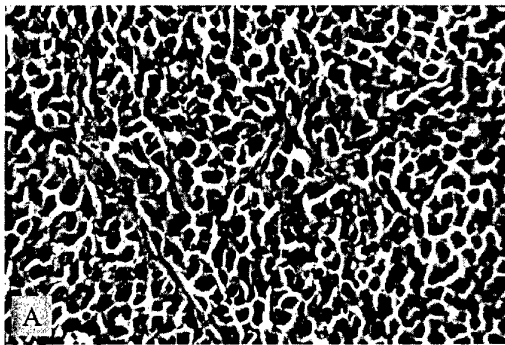


Figure 4: GDEPT effected by RM1CDUPRT cells *in vivo*.

5x10³ of RM1-GFP/CDUPRT (test) or RM1-GFP/LacZ (control) cells were implanted orthotopically in the prostate of C57BL/6 mice. 4 days post-implantation the prodrug 5FC (or saline, as a control) was administered intraperitoneally at 500mg/kg/mouse/day for 13 days. On day 19, mice were sacrificed and their prostate tumour volumes determined using the formula, $V = \pi/6(d_1 \cdot d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles.

H & E staining of paraffin-embedded RM1-CDUPRT cells grown in the prostate of C57BL/6 mice.

Saline



5 FC

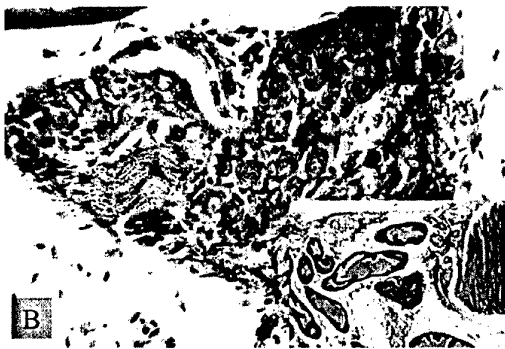


Figure 5: Effects of CDUPRT-GDEPT on histology of RM1 tumors

H & E staining of paraffin-embedded iprost RM1-CDUPRT prostate tumor sections. Treatment with saline resulted in highly vascularised viable tumor (A x40 and insert, x10), whereas treatment with 5FC resulted in necrosis (B x40) with loss of prostate tissue architecture (insert, x10).

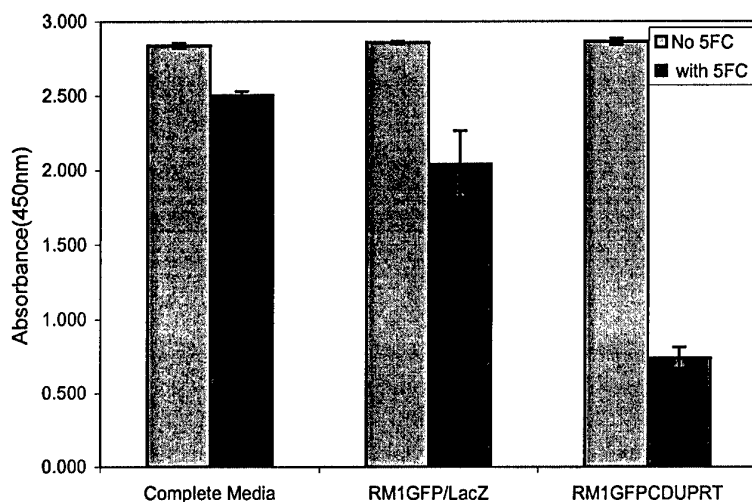


Figure 6A: *In vitro* evaluation of the local bystander effect of CDUPRT-GDEPT. RM1-GFP/CDUPRT cells were grown in presence or absence of 5FC for 48 hrs, at the end of which the supernatants were collected. Supernatants from RM1-GFP/LacZ cells grown in the presence of 5FC, served as the controls. Parental RM1 cells were then treated with these supernatants at 50% concentration. The bystander effect was demonstrated by cell killing of parental RM1 cells using the conditioned media from RM1CDUPRT cells.

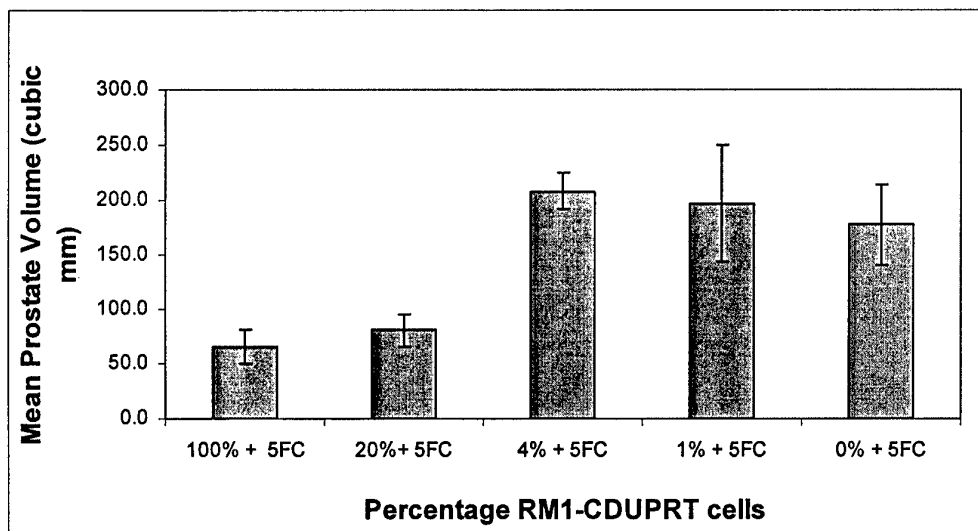


Figure 6B: Local bystander effect of CDUPRT-GDEPT *in vivo*. RM1-GFP/CDUPRT cells were mixed with RM1-/GFP cells in different proportions (as shown) and a total of 5×10^3 cells were implanted in the prostate of C57Bl/6 mice. The mice were injected intraperitoneally with the prodrug 5FC at 500mg/kg/mouse/day from day 4 onwards daily for 15 days. Mice were sacrificed on day 19, and their prostate volumes determined using the formula, $V = \pi/6(d_1 \cdot d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles.

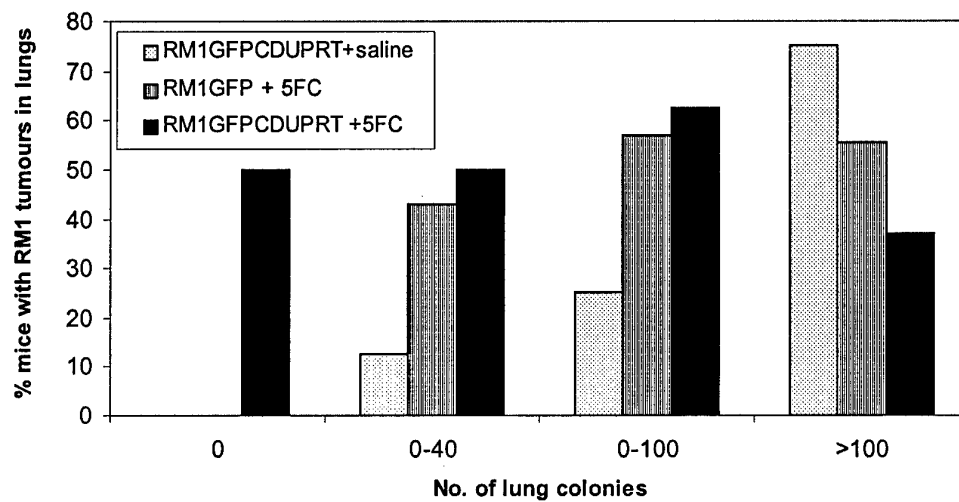


Figure 7: Distant Bystander effect of CDUPRT-GDEPT *in vivo*.

RM1CDUPRT (5×10^3) cells were implanted in the prostate of C57Bl/6 mice. Four days later, the mice were injected intravenously with the parental RM1 cells at the dose of 2.5×10^5 cells/mouse. The mice were injected ip with the prodrug 5FC at from day 4 onwards daily for 15 days 500mg/kg/mouse/day. On day 19, the mice were sacrificed and the lungs were harvested, stored in Bouin's reagent and colony counts were performed.

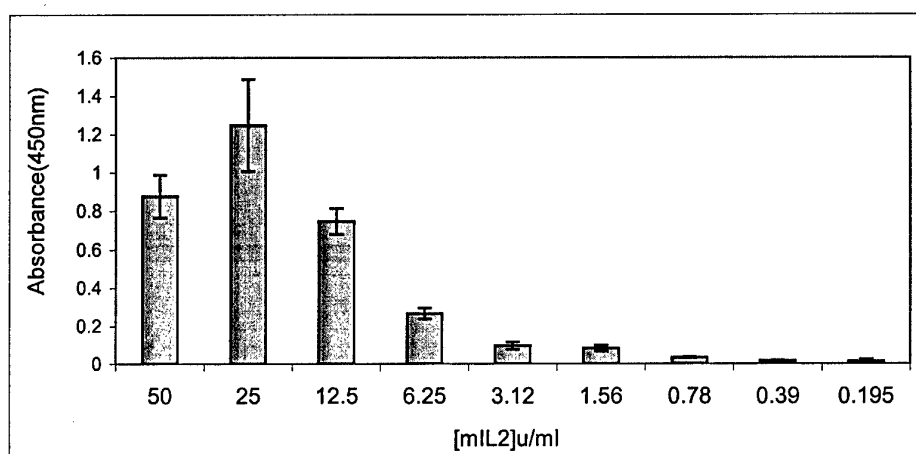


Figure 8: Proliferation of CTLL2 cells in response to mIL2 at 72 hr post-treatment.

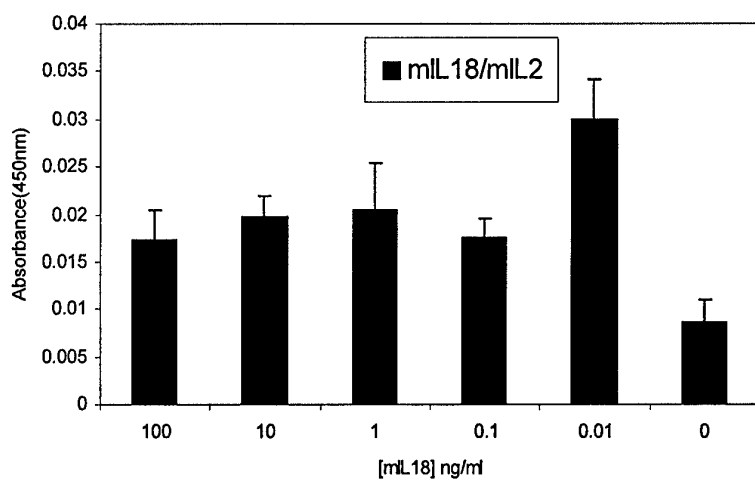
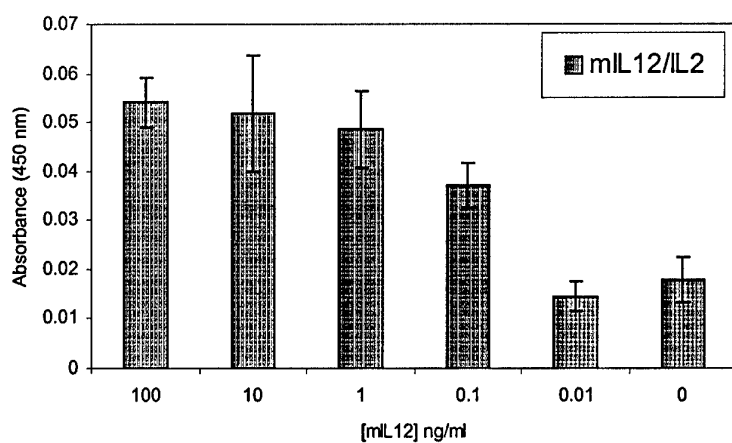


Figure 9: CTLL2 proliferation in response to recombinant mIL12 (A) and mIL18 (B).

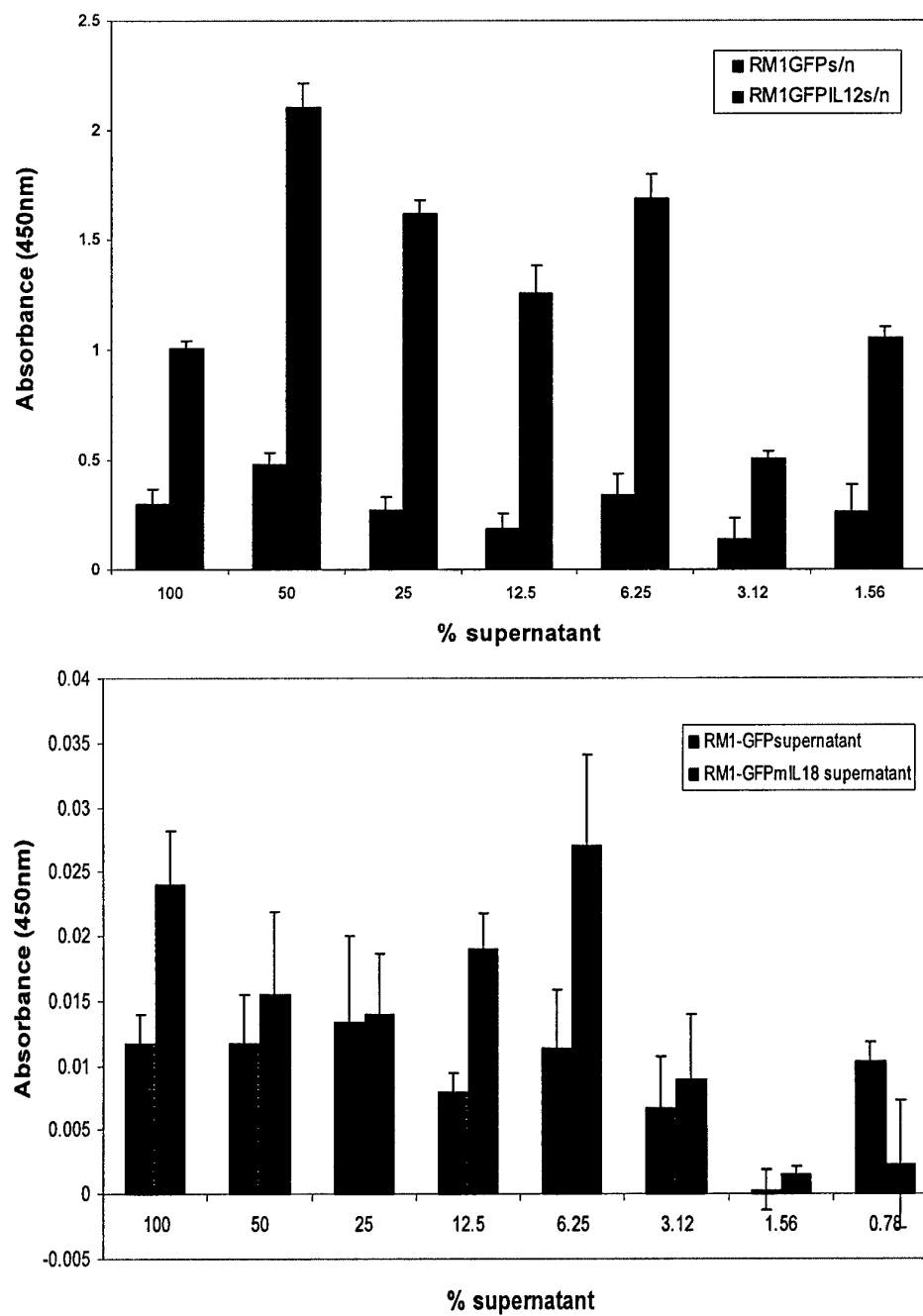


Figure 10: Effects of supernatants from RM1-GFPmIL12 (A) and RM1-GFPmIL18 cells (B) on CTLL2 proliferation. Supernatants from RM1-GFP cells were used as the controls.

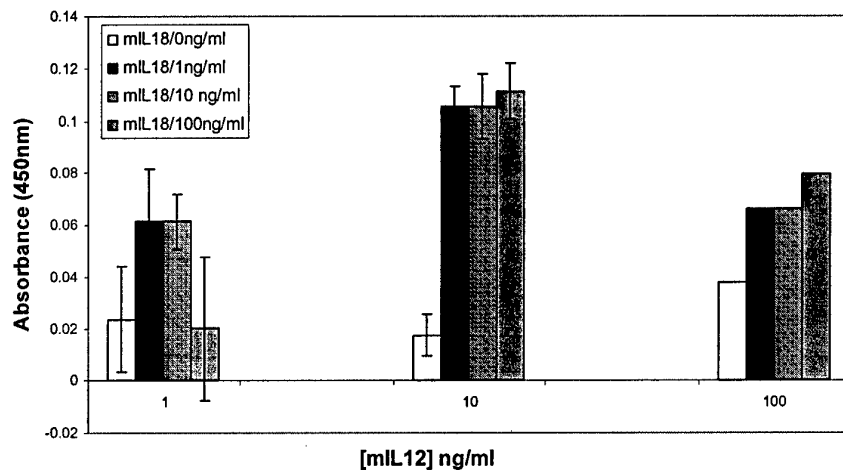
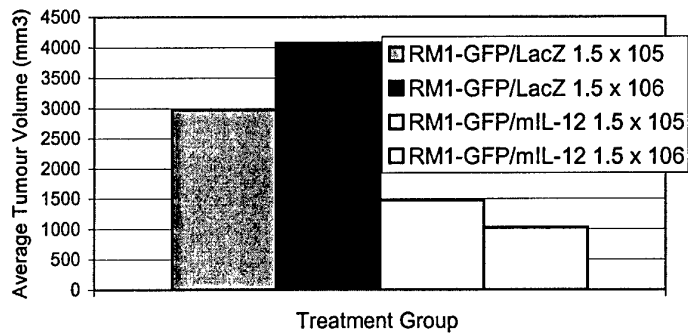


Figure 11: CTLL2 proliferation in response to combination of recombinant mIL12 and mIL18. Cells were incubated with mIL12 for 24 hrs followed by addition of mIL18, and then analysed for proliferation 48 hrs later. The data are normalised for no addition of mIL12 or mIL18.

A

RM1-GFP/mL-12 SC growth Day 14



B

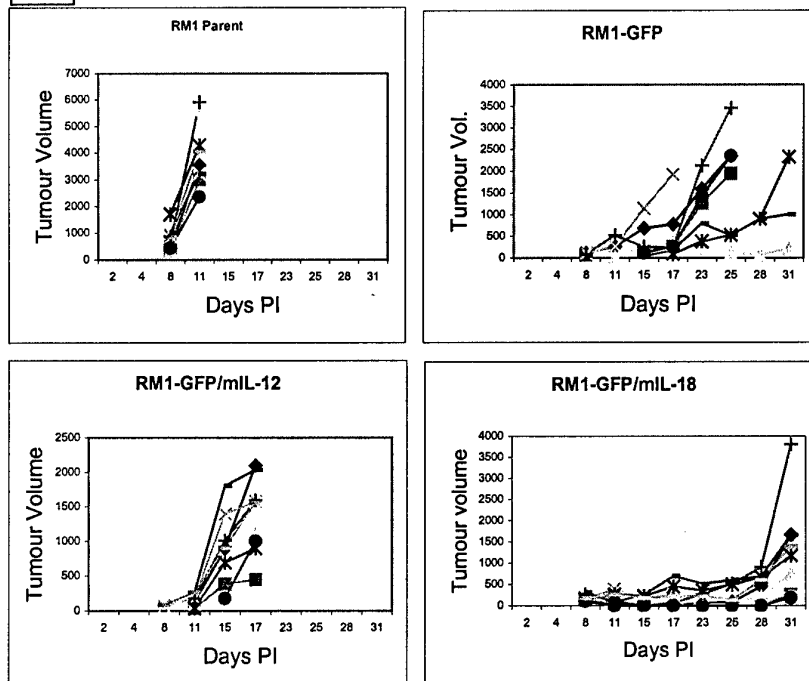


Figure 12: Growth of RM1-GFP/IL12 or RM1-GFP/IL18 cells sc in C57BL/6 mice.

A: shows mean tumor volumes indicating that higher numbers of RM1-GFP/IL12 cells grew to a smaller volume than those of lower numbers, suggesting that IL-12 secretion was slowing tumor growth.

B: shows the tumour volume achieved in individual mice after injecting 3×10^5 cells sc. PI = post injection.

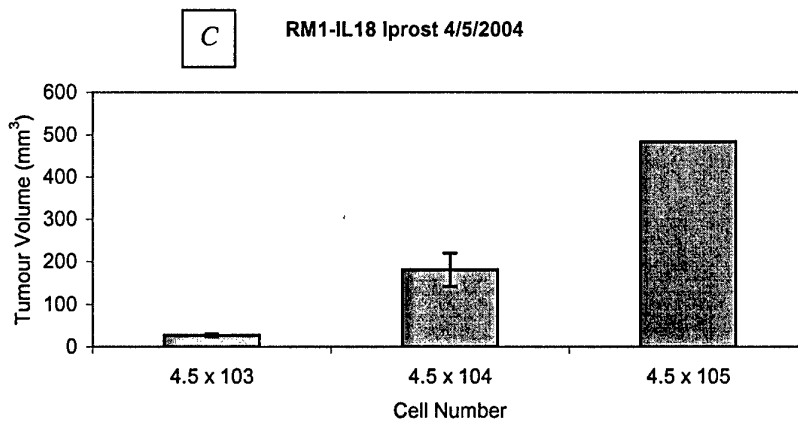
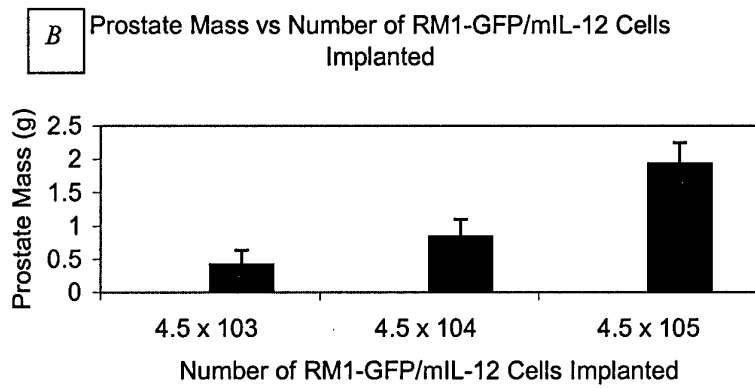
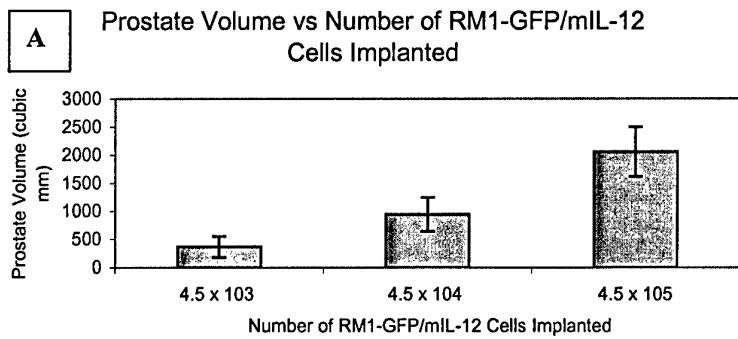


Figure 13: Relative growth of RM1-GFP/IL12 cells (A, prostate volume; B prostate mass) or C RM1-GFP/IL18 cells in the prostate of C57BL/6 mice after orthotopic implantation.

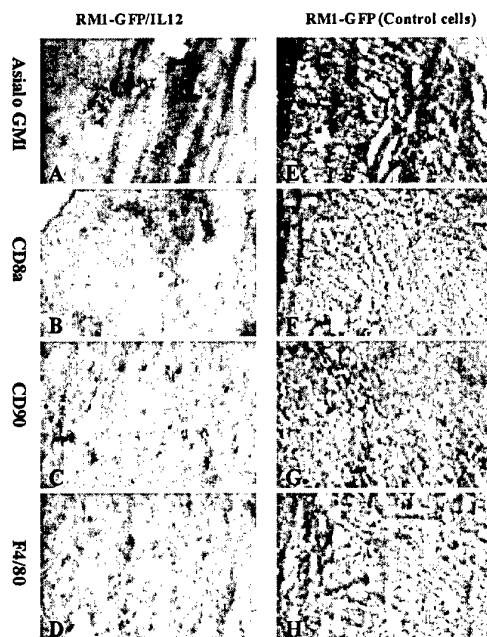


Figure 14: Photomicrographs of immune reactive cell infiltration (x10) in RM1 tumors expressing IL12.

Immunoreactivity (brown staining) to Asialo-GM1, CD8, CD90 and F4/80 of infiltrating immune cells in frozen tumor sections of RM1-GFP/IL12 cells (A-D) and RM1-GFP (control cells, E-H) grown in the prostate of C57BL/6 mice. A and E are stained for Asialo-GM cells; B and F for CD8a, C and G for CD90 and D and H for F4/80 positive cells. RM1 tumors expressing IL12 show increased infiltration of Asialo-GM+ (A) and CD90 cells (C) compared with control cells (E and G), as well as of CD8a (B) and F4/80 (D) positive cells that are absent in RM1-GFP tumors (F and H).